Efficient *In Vivo* Delivery of siRNA to the Liver by Conjugation of α-Tocopherol

Kazutaka Nishina[1,2], Toshinori Unno[1], Yoshitaka Uno[1], Takayuki Kubodera[1,2], Tadashi Kanouchi[1], Hidehiro Mizusawa[1,2] and Takanori Yokota[1]

[1]*Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan;* [2]*21st Century Center of Excellence Program on Brain Integration and Its Disorders, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan*

RNA interference is a powerful tool for target-specific knockdown of gene expression. However, efficient and safe *in vivo* delivery of short interfering RNA (siRNA) to the target organ, which is essential for therapeutic applications, has not been established. In this study we used α-tocopherol (vitamin E), which has its own physiological transport pathway to most of the organs, as a carrier molecule of siRNA *in vivo*. The α-tocopherol was covalently bound to the antisense strand of 27/29-mer siRNA at the 5′-end (Toc-siRNA). The 27/29-mer Toc-siRNA was designed to be cleaved by Dicer, producing a mature form of 21/21-mer siRNA after releasing α-tocopherol. The C6 hydroxyl group of α-tocopherol, associated with antioxidant activity, was abolished. Using this new vector, intravenous injection of 2 mg/kg of Toc-siRNA, targeting *apolipoprotein B* (*apoB*), achieved efficient reduction of endogenous *apoB* messenger RNA (mRNA) in the liver. The downregulation of *apoB* mRNA was confirmed by the accumulation of lipid droplets in the liver as a phenotype. Neither induction of interferons (IFNs) nor other overt side effects were revealed by biochemical and pathological analyses. These findings indicate that Toc-siRNA is effective and safe for RNA interference–mediated gene silencing *in vivo*.

Received 13 October 2007; accepted 7 January 2008; published online 12 February 2008. doi:10.1038/mt.2008.14

INTRODUCTION

Short interfering RNAs (siRNAs) have potential for therapeutic application in a wide spectrum of disorders including cancer, infectious diseases, and inherited diseases. Effective *in vivo* delivery of siRNAs to the specific target cells is the most important challenge in respect of clinical applications. *In vivo* gene silencing with RNA interference has been reported using either viral vectors[1] or high-pressure, high-volume intravenous injection of synthetic siRNAs,[2,3] but these approaches have limitations in clinical practice because of their side effects. Accordingly, a variety of nonviral systems are being developed for delivery of siRNA to liver, tumors, and other tissues *in vivo*.

Recent work in the area of nonviral delivery of synthetic siRNAs has used cationic liposomes[4–6] or nanoparticles.[7] Among these approaches, the most efficient systemic administration was achieved using stable nucleic acid lipid particles.[4] However, a therapeutic dose (2.5 mg/kg) of these particles, when administered in cynomolgus monkeys, caused marked liver damage.[4] A key drawback of cationic liposomes and nanoparticles is that their physical lipophilic property promotes passive transfer of siRNA complexes to the liver, potentially causing toxicity. More recently, a new class of receptor-mediated siRNA vectors, consisting of a synthetic compound and a ligand, has been reported. These ligands are (i) *N*-acetylgalactosamine[8] or galactose[9] ligands that target asialoglycoprotein receptors on hepatocytes, (ii) apolipoprotein A-I ligands that target scavenger receptor class B type I on the hepatocytes,[10] and (iii) rabies virus glycoprotein ligands that target acetylcholine receptors on the neurons.[11] These receptor-mediated delivery systems could increase efficiency and specificity of target cells *in vivo*. However, the synthetic molecules of these vectors were found to exert an immunostimulatory effect.[8]

We hypothesized that the most effective *in vivo* carrier would be a molecule that is essential for target tissue cells but cannot be synthesized within the cells. Vitamins fit these requirements well, and the only vitamin that is not toxic even at high doses is vitamin E.[12] α-Tocopherol (vitamin E) is a fat-soluble natural molecule that has many physiological pathways from serum to liver. The majority of the absorbed vitamin E is transferred into lipoproteins including chylomicrons, low-density lipoprotein, and high-density lipoprotein, and these constitute an important source of plasma vitamin E for hepatic uptake (reviewed in ref. 13). In addition, the three α-tocopherol-associated proteins (SEC14L2, SEC14L3, and SEC14L4), and the albumin-related protein, afamin, are known to be vitamin E–binding proteins in the serum (reviewed in ref. 14). In this study, we have tried to utilize these physiological pathways of vitamin E transport to the liver as an *in vivo* delivery system for siRNA.

RESULTS

Design of α-tocopherol-bound siRNA

Asymmetric double-strand RNA having 2 nucleotides (nt) in 3′-overhang only in the antisense strand is good for predicting a

The first two authors contributed equally to this work.

Correspondence: Takanori Yokota, Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: tak-yokota.nuro@tmd.ac.jp

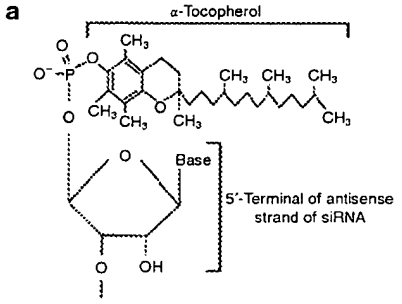

Figure 1 Design of α-tocopherol-bound short interfering RNA (siRNA). (**a**) Chemical structure of vitamin E (α-tocopherol)-bound siRNA. (**b**) Sequences and chemical modifications of α-tocopherol-bound siRNA for targeting *apoB* messenger RNA (apoB-1 Toc-siRNA) or for targeting unrelated gene (control Toc-siRNA). The lower-case letters represent sugar 2′-O-methylation, and asterisks represent phosphorothioate backbone linkage. The predicted cleavage sites by Dicer[15] and Argonaute2 (ref. 19) are indicated by black bars and arrowheads, respectively. The sequences in bold letters indicate the predicted 21-mer siRNA sequences after Dicer cleavage. Toc; α-tocopherol.

Dicer cleavage site and can therefore define the 21-mer siRNA sequence cleaved from 27/29-mer siRNA by Dicer.[15] The α-tocopherol was covalently bound to the 5′-end of the antisense strand of these siRNAs. The chemical structure of α-tocopherol-bound siRNA (Toc-siRNA) is shown in Figure 1a. The sequences of (i) Toc-siRNA for targeting mouse *apolipoprotein B* (*apoB*) messenger RNA (mRNA) (NM_009693) (apoB-1 Toc-siRNA)[16] and (ii) Toc-siRNA for targeting mouse *beta-site APP cleaving enzyme 1* (*BACE1*) mRNA (NM_011792) (control Toc-siRNA) are shown in Figure 1b.

For *in vivo* application of Toc-siRNA, it is essential to ensure the stability of siRNA against serum-derived nucleases. For this purpose, we made chemical modifications with phosphorothioate backbone linkage and sugar 2′-O-methylation on both the sense and the antisense strands. The portions of siRNA that were predicted to be cleaved out by Dicer, *i.e.*, 8 nt in the 5′-side of the antisense strand and 6 nt in the 3′-side of the sense strand, were substantially modified. Further, in order to increase stability against endonucleases while preserving siRNA activity,[17] partial internal modifications were made to the siRNA sequences with 2′-O-methylation, in addition to modifications at the termini.[18] The Dicer cleavage sites in both sense and antisense strands, and the Argonaute2 cleavage site[19] in the sense strand were spared any modification (Figure 1b).

Improved stability of siRNA with preserved cleaving efficiency by chemical modifications

The naked and the chemically modified siRNA in serum were compared for stability *in vitro*. With and without α-tocopherol,

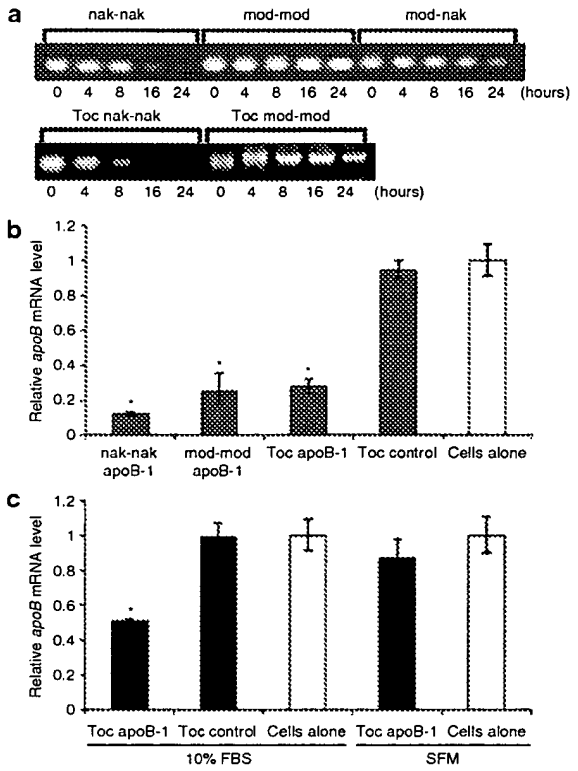

Figure 2 Improvement in stability and preservation of cleaving efficiency of short interfering RNA (siRNA) after chemical modification. (**a**) The stability of modified siRNA in the serum. The both-strands-naked siRNA (nak-nak), both-strands-modified siRNA (mod-mod), only-sense-strand-modified siRNA (mod-nak), both-strands-naked α-tocopherol-bound siRNA (Toc nak-nak), and both-strands-modified α-tocopherol-bound siRNA (Toc mod-mod) were incubated in the mouse serum at 37 °C for 4, 8, 16, and 24 hours. The samples were treated with Proteinase K and electrophoresed in 2% agarose gel. (**b**) Reduction of *apoB* messenger RNA (mRNA) levels in the Hepa 1-6 cell line after transfection with apoB-1 siRNA using Lipofectamine RNAiMAX. The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses of *apoB* mRNA levels relative to *gapdh* mRNA were performed 24 hours after transfection of both-strand-naked apoB-1 siRNA (nak-nak apoB-1), both-strand-modified apoB-1 siRNA (mod-mod apoB-1), both-strand-modified apoB-1 Toc-siRNA (Toc apoB-1), and control Toc-siRNA (Toc control). The data shown are relative to the values in untreated cells (Cells alone). $n = 3$, Data are shown as mean values ± SEM. $^*P < 0.005$ as compared to cells-alone group. (**c**) Reduction of *apoB* mRNA levels in the Hepa 1-6 cell line after transfection using apoB-1 Toc-siRNA alone. The qRT-PCR analyses of *apoB* mRNA levels relative to *gapdh* mRNA were performed 24 hours after transfection with apoB-1 Toc-siRNA (Toc apoB-1) and control Toc-siRNA (Toc control). The Hepa 1-6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) only [serum-free medium (SFM)], or in DMEM supplemented with 10% fetal bovine serum (10% FBS). The data shown are relative to the values in untreated cells (Cells alone). $n = 3$, Data are shown as mean values ± SEM. $^*P < 0.005$ as compared to cells-alone group. Toc; α-tocopherol. *gapdh, glyceraldehyde-3-phosphate dehydrogenase.*

the stability of the siRNA with both strands modified was much greater than those of the siRNA with both strands naked, and the siRNA with only the sense strand modified. The conjugation of α-tocopherol did not increase the stability of siRNA (Figure 2a).

The impact of the silencing ability conferred by the chemical modification of siRNAs and binding of α-tocopherol was studied in cultured cells of mouse hepatocellular carcinoma (Hepa 1-6)

using a transfection reagent. Even with considerable chemical modification of both strands, the silencing effect of apoB-1 siRNA on endogenous *apoB* mRNA in the Hepa 1-6 cells was not much impaired when compared with the silencing effect of apoB-1 siRNA with both strands naked. Further, the binding of α-tocopherol to the apoB-1 siRNA with both strands modified also did not interfere with the silencing activity (Figure 2b). In effect, we succeeded in carrying out considerable appropriate chemical modifications in the siRNA sequences to increase serum stability, while preserving silencing activity.

Next, α-tocopherol-mediated induction of siRNA was confirmed in Hepa 1-6 cells without any transfection reagents. The addition of apoB-1 Toc-siRNA to the culture medium reduced endogenous *apoB* mRNA in Hepa 1-6 cells. This silencing effect disappeared when serum was absent in the cultured medium (Figure 2c). This finding suggests that α-tocopherol can introduce siRNA into the cells in association with molecules in the serum.

Effective delivery and processing of Toc-siRNA in mice liver

In order to investigate whether successful delivery of Toc-siRNA had been achieved, liver sections were taken from mice 1 hour after injection with Cy3-labeled Toc-siRNA (Cy3 bound to the sense strand of siRNA), and the sections were subjected to confocal imaging. We observed marked accumulation of Cy3 signal both in hepatocytes and nonparenchymal cells in the liver sinusoids. Almost all the hepatocytes had the Cy3 signal. There was predominant signal density around central veins. There was no Cy3 signal in the control liver sections from the mouse injected with Cy3-labeled siRNA without α-tocopherol (Figure 3a). We also confirmed a less prominent Cy3 signal in other organs including lung; the details of the systemic distribution of Toc-siRNA are to be published elsewhere.

In order to study whether Toc-siRNA is processed to a mature form of 21/21-mer siRNA, northern blotting was performed on mouse liver after injection of 32 mg/kg Toc-siRNA. The assay showed two bands of sizes ~21 nt and ~29 nt, corresponding to the processed 21-mer antisense strand and the 29-mer α-tocopherol-bound antisense strand, respectively (Figure 3b). These results clearly show that Toc-siRNA has the ability to enter mouse liver cells and be processed by Dicer in the cytosol.

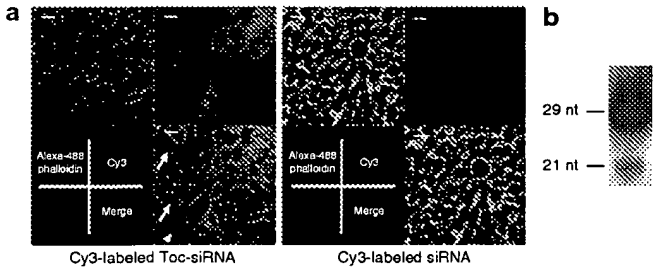

Figure 3 Targeted delivery of Toc-siRNA to mice livers after injection. **(a)** Confocal images of liver sections from mice injected intravenously with Cy3-labeled Toc-siRNA (left panel) and Cy3-labeled siRNA (right panel). Cy3 signal (red) was noted in hepatocytes (arrowhead) and nonparenchymal cells (arrows). Liver sections were stained with Alexa-488 phalloidin to visualize cell outlines (green). Scale bar = 20 μm. **(b)** Small RNAs isolated from livers of apoB-1 Toc-siRNA-injected mice were probed with siRNA sense strand oligonucleotide in order to examine for the presence of apoB-1 Toc-siRNA antisense strand using northern blotting. The bands for the 21 nucleotides (nt) as well as the 29–30-nt antisense strands were detected. siRNA, short interfering RNA; Toc; α-tocopherol.

Knockdown of target genes in liver and phenotypic analyses of mice using Toc-siRNA

In order to assess the silencing ability of Toc-siRNA *in vivo*, the level of endogenous *apoB* mRNA in the liver was evaluated. The liver was removed 48 hours after the injection and assayed for *apoB* mRNA levels using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The 2 mg/kg apoB-1 Toc-siRNA markedly suppressed *apoB* mRNA when compared with the effect produced by the same volume of maltose, and this silencing effect disappeared when α-tocopherol was not bound to the siRNA. The knockdown effect was specific for the target molecule, as evidenced by the finding that other endogenous mRNAs in the liver, *transthyretin* (*ttr*) and *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), did not change, and that a control Toc-siRNA targeting an unrelated gene did not affect them, when mRNA levels were measured relative to total RNA (Figure 4a).

Next, we performed a time-course experiment to determine the duration of *apoB* mRNA knockdown effect after single injection of apoB-1 Toc-siRNA. After injection, the reduction of *apoB* mRNA in liver was maximal on day 1 and gradually returned to the baseline level on day 4 (Figure 4b). We also performed a dose-response experiment on day 2 after injection. Mice treated with 2, 8, and 32 mg/kg of apoB-1 Toc-siRNA showed significant does-dependent reduction in *apoB* mRNA levels (Figure 4c). The intestine, another organ where *apoB* is expressed, was also removed 24 hours after injection and assayed for *apoB* mRNA levels using qRT-PCR. There was no knockdown effect in the intestine as a result of the apoB-1 Toc-siRNA injection (data not shown).

The reduction in liver *apoB* mRNA lowered the export of very low-density lipoprotein (VLDL) from the liver, resulting in a decrease of serum triglyceride (TG) and cholesterol levels and an increase in hepatic lipids.[8] Injection of Toc-siRNA produced significant reduction in TG and cholesterol levels on day 1 (Figure 5a and b). Further, we performed pathological analysis using Sudan III lipid-staining of liver tissue. The liver sections from mice injected with 2 mg/kg of apoB-1 Toc-siRNA showed a higher number of hepatic lipid droplets than liver sections from control Toc-siRNA-injected mice (Figure 5c). Taken together, these results indicate that apoB-1 Toc-siRNA inhibits *apoB* mRNA and alters the phenotype of lipid metabolism in the liver.

No side effects are produced by Toc-siRNA

White blood cell and platelet counts and biochemical analysis of the serum including total protein, aminotransaminases, and blood urea nitrogen after the injection of 2 mg/kg Toc-siRNA (Table 1), and pathological analysis of the liver tissue stained with hematoxylin/eosin (data not shown) did not show any marked abnormalities.

The level of induction of interferons (IFNs) was examined at 3 hours (the time interval known to be the IFN phase) after the injection of Toc-siRNA.[5] No IFN-α was detected in the serum (Table 1), and RT-PCR of the liver RNA did not amplify *IFN-β*

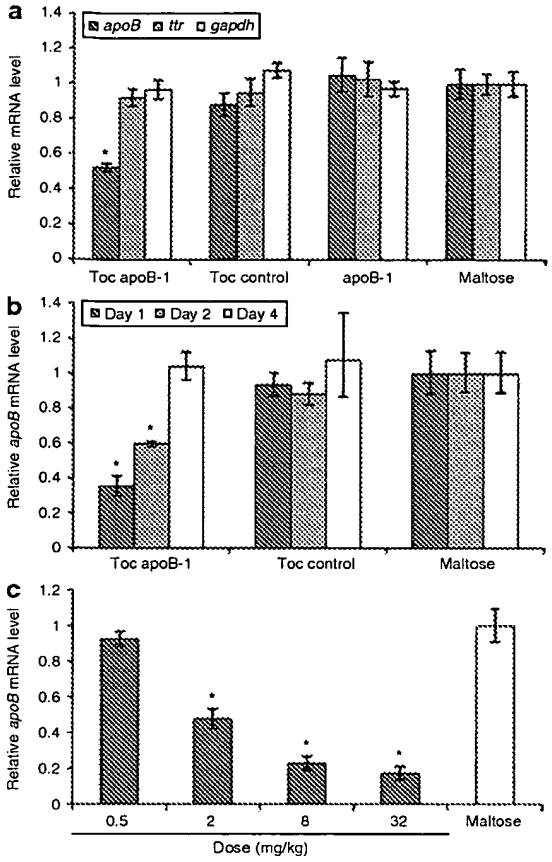

Figure 4 Toc-siRNA-mediated silencing of mouse *apoB* messenger RNA (mRNA) in liver is potent, specific, and dose-dependent. (**a**) The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses of several endogenous mRNAs, *apoB, ttr,* and *gapdh* mRNAs in the liver (removed 2 days after injection) relative to total input RNA. $n = 3$, The data shown are mean values ± SEM. $^*P < 0.005$ as compared to the maltose injection group. (**b**) Duration of the gene silencing caused by apoB-1 Toc-siRNA. The qRT-PCR analyses of liver *apoB* mRNA levels relative to *gapdh* mRNA were performed at the indicated time points after injection of apoB-1 Toc-siRNA (Toc apoB-1) or control Toc-siRNA (Toc control). $n = 3$, The data shown are mean values ± SEM. $^*P < 0.005$ as compared to the maltose injection group. (**c**) Dose-dependent reduction of *apoB* mRNA levels in the liver after injection of apoB-1 Toc-siRNA. The *apoB* mRNA levels (normalized to *gapdh* mRNA) were determined 2 days after injection of apoB-1 Toc-siRNA quantitated by qRT-PCR. The data shown are relative to those of mice receiving maltose alone. $n = 3$, The data shown are mean values ± SEM. $^*P < 0.005$ as compared to the maltose injection group. siRNA, short interfering RNA; Toc; α-tocopherol. *gapdh, glyceraldehyde-3-phosphate dehydrogenase; ttr, transthyretin.*

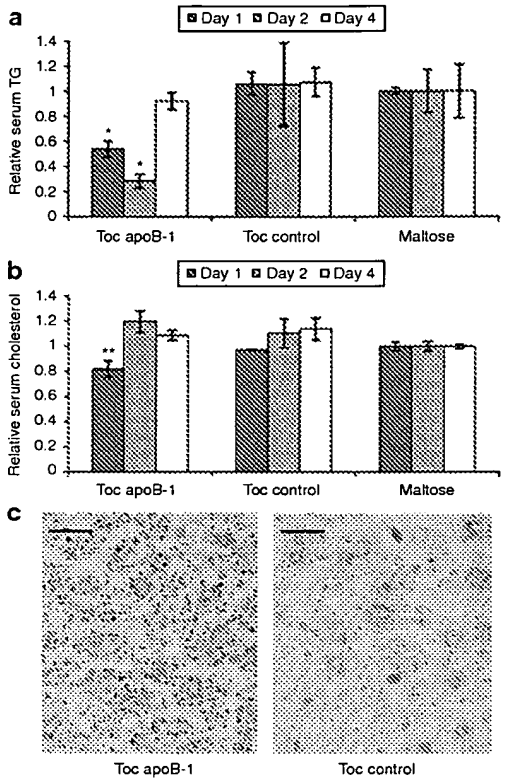

Figure 5 Phenotypic change in lipid metabolism caused by inhibition of liver *apoB* messenger RNA (mRNA). Decreased levels of (**a**) serum triglyceride (TG) and (**b**) cholesterol after knockdown of *apoB* mRNA by apoB-1 Toc-siRNA. Sera were collected from mice before the injections and at 24, 48, and 96 hours after the injections of apoB-1 Toc-siRNA (Toc apoB-1) or control Toc-siRNA (Toc control). The sera were analyzed for TG and cholesterol levels. The values obtained after the injections were divided by those obtained before the injections, and the resultant ratios were normalized to mice which treated with maltose injection. $n = 3$, The data shown are mean values ± SEM. $^*P < 0.01$, $^{**}P < 0.05$ as compared to the maltose injection group. (**c**) Reduction in *apoB* mRNA results in increased hepatic lipid accumulation. Liver sections were prepared 4 days after injection of apoB-1 Toc-siRNA and control Toc-siRNA. The sections were fixed, and lipids were detected by staining with Sudan III. Scale bar = 2μm. siRNA, short interfering RNA; Toc; α-tocopherol.

mRNA (data not shown). The chemical modifications have been reported as preventing stimulation of Toll-like receptor in the endosomes when siRNA is delivered with cationic liposomes.[20,21] However, the absence of an IFN response to Toc-siRNA does not seem to be the result of chemical modification alone; indeed, a 2 mg/kg dose of Toc-siRNA without chemical modifications also did not induce IFNs (data not shown).

DISCUSSION

We hypothesized that the most effective *in vivo* carrier of siRNA would be a molecule that is essential for target tissue cells but cannot be synthesized within the cells. Vitamins fit these requirements well, and the least toxic of the vitamins even at high doses is vitamin E.[12] Among the eight natural isomers of vitamin E, α- and γ-tocopherol are the most abundant in human diets and are equally well absorbed, but peripheral tissues contain much more of α-tocopherol than of γ-tocopherol,[22] thereby indicating the presence of a selective transport system for α-tocopherol. We therefore planned to use α-tocopherol and its transport system to effect the delivery of siRNA. Because (hydrophilic) siRNA and (lipophilic) α-tocopherol cannot be admixed, we directly bound α-tocopherol molecule to siRNA at the 5′-end of the 29-mer siRNA antisense strand with a phosphate bond (Toc-siRNA) (Figure 1a and b). We designed 27/29-mer Toc-siRNA with 2 nt 3′-overhang of the antisense strand. The α-tocopherol with 6/8-mer double-strand RNAs is to be cleaved by Dicer in the cytosol, generating the mature form of 21/21-mer siRNA (Figure 1b). We actually confirmed, using northern blotting, that the processed 21-mer siRNA antisense strand was detected in mouse liver after injection with Toc-siRNA (Figure 3b), and that the binding of

Table 1 IFN-α, BUN, TP, AST, ALT, WBC, and Plt levels in mouse serum after intravenous injection of 2mg/kg apoB-1 Toc-siRNA or maltose

Treatment		IFN-α (pg/ml)	BUN (mg/dl)	TP (g/dl)	AST (U/l)	ALT (U/l)	WBC (/μl)	Plt (×10⁴/μl)
apoB-1 Toc-siRNA	3 hours	<12.5						
	24 hours		19.1 ± 1.0	5.1 ± 0.1	78 ± 1	21 ± 3	2,800 ± 330	122.0 ± 0.3
	48 hours		24.0 ± 2.4	5.5 ± 0.1	67 ± 4	22 ± 1	2,600 ± 550	112.2 ± 18.9
maltose	3 hours	<12.5						
	24 hours		22.0 ± 0.9	5.5 ± 0.1	79 ± 9	25 ± 2	2,600 ± 560	117.9 ± 13.8
	48 hours		24.5 ± 1.5	5.5 ± 0.1	60 ± 3	26 ± 3	3,700 ± 900	109.0 ± 7.0

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; IFN-α, interferon-α; Plt, platelet; TP, total protein; WBC, white blood cell.
The values shown are mean values ± SEM (n = 3).

α-tocopherol did not interfere with the siRNA activity *in vitro* (Figure 2b).

This study showed that the binding of α-tocopherol to siRNA enables the efficient *in vivo* delivery of siRNA to the liver. The direct conjugation to siRNA of another lipophilic molecule, cholesterol (Chol-siRNA), was also reported to enhance liver uptake of siRNA.[16] However, the silencing effect produced by Toc-siRNA was more efficient than that by Chol-siRNA, in relation to the identical target gene; much higher doses of Chol-siRNA (50–100 mg/kg) were required for achieving an efficient reduction of *apoB* mRNA in the liver.[4,16] Actually, when cholesterol was conjugated to the same 27/29-mer apoB-1 siRNA with the same chemical modifications as used in apoB-1 Toc-siRNA at the 3′-end of the sense strand, the reduction of *apoB* mRNA induced by 2 mg/kg of this Chol-siRNA was not statistically significant in the livers of mice (data not shown).

The mechanism of uptake of Toc-siRNA by the liver was not elucidated, and the cause of the difference in silencing efficiency between Toc-siRNA and Chol-siRNA is not known. However, there are some possible explanations. First, if α-tocopherol and cholesterol fuse into the lipid bilayer of hepatocyte membrane as cationic liposome does, the difference in hydrophobicity and polarity between α-tocopherol and cholesterol might influence the efficiency of uptake of siRNA by the liver. This cannot be proved, however, because the negative charge of siRNA cannot be cancelled by the addition of α-tocopherol or cholesterol. Moreover, our *in vitro* experiments indicated that Toc-siRNA does not enter the hepatoma culture cell without serum. Second, Toc-siRNA might be incorporated into the serum lipoproteins and enter the hepatocytes via lipoprotein receptors. Recently, Chol-siRNA was shown to use the lipoprotein receptor–mediated pathway to enter hepatocytes.[23] In contrast to cholesterol, α-tocopherol is an exogenous lipid which cannot be synthesized *in vivo*, and therefore the distribution of α-tocopherol among lipoproteins and the mediating receptors in the liver might be different from those of cholesterol. Third, binding of α-tocopherol might enhance uptake of siRNA in the liver by an interacting serum molecule other than lipoprotein. Soutschek and colleagues[16] proposed that the mechanism of Chol-siRNA *in vivo* is related to enhanced binding to serum protein such as albumin. Similarly, α-tocopherol is known to interact with other serum proteins such as SEC14L2, SEC14L3, SEC14L4, and afamin (reviewed in ref. 14).

We observed significant decreases of serum TG and cholesterol and an increase in lipid droplets in the liver after injection of apoB-1 Toc-siRNA. The downregulation of liver ApoB-100 impairs VLDL export and is expected to decrease serum TG as well as cholesterol, because large amounts of TG are incorporated into VLDL particles. This is supported by the fact that the transgenic mouse of truncated *apoB*,[24] and the microsomal TG transfer protein-null mouse,[25,26] neither of which can assemble and secrete VLDL in the liver, show lower serum TG and cholesterol and an accumulation of lipid droplets in the liver. Our results, showing decrease in serum TG as well as in cholesterol, were similar to those of a recent study that used a different siRNA *in vivo* delivery system.[8] Although the decreases in serum TG and cholesterol might be caused by mechanisms other than impaired VLDL export, these results indicate the phenotype of ApoB-100 silencing by Toc-siRNA.

There was no remarkable side effect in blood cell count and biochemical analysis after intravenous injection of Toc-siRNA. The delivered amount of α-tocopherol was only 46 μg/kg when 2 mg/kg Toc-siRNA was injected. This value is very small, considering the need of α-tocopherol as a nutritional element is estimated 10 mg/day for man (125–200 μg/kg/day).[27] In addition, the anti-oxidant activity of α-tocopherol in Toc-siRNA is abolished, because the reactive site of α-tocopherol for anti-oxidation, hydroxyl group at the C6 position, is covalently connected to siRNA (Figure 1a). More important, the Toc-siRNA did not induce IFN-α in serum (Table 1) and *IFN-β* mRNA in the liver. This absence of adverse side effects associated with the use of Toc-siRNA is important to note, because it is in sharp contrast to the outcome generally described for lipid vector–associated siRNA delivery. The latter is known to produce an immunostimulatory effect,[28] which could cause elevation of transaminases, thrombocytopenia, and lymphopenia.[29] When synthetic lipid–coated siRNA is intravenously injected, it is incorporated in the endosome and then induces IFNs and cytokines through activation of Toll-like receptors located in the endosomal membrane.[20] The possible mechanism of escape from an immunostimulatory effect in Toc-siRNA-injected mice was that Toc-siRNA used the different pathway to enter the cells from synthetic lipid–coated siRNAs. Together, Toc-siRNA is considered to be a noninvasive delivery method of siRNA.

In summary, vitamin E–mediated *in vivo* delivery of siRNA is effective and safe. Although further investigation into the precise delivery pathway of Toc-siRNA is required for better optimization of its use, the findings of this study represent an important step in advancing the use of synthetic siRNA as a very promising system for gene therapy.

MATERIALS AND METHODS

Synthesis of siRNAs. siRNAs were chemically synthesized. In order to combine vitamin E with siRNA, α-tocopherol phosphoramidite was prepared, and then was it connected with the 5′-end of the antisense strand of the siRNA. The DL-α-tocopherol was purchased from Tokyo Kasei, Tokyo, Japan. Synthetic sense and antisense strands of siRNA were then annealed.

Cell culture. Hepa 1-6 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) only, or supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg of streptomycin at 37 °C in 5% CO_2.

qRT-PCR. Total RNA was extracted from the culture cells or mice liver using Isogen (Nippon Gene, Tokyo, Japan). The RNA was reverse transcribed with Superscript III and random hexamers (Invitrogen, Carlsbad, CA). The qRT-PCR was performed on 1.5 μg of complementary DNA using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. The amplification conditions were 40 cycles of denaturation at 95 °C for 15 seconds and annealing at 60 °C for 60 seconds with ABI PRISM 7700 Sequence Detector. Primers for mouse *apoB*, *gapdh*, *ttr*, and *IFN-β* mRNAs were designed by Applied Biosystems (Foster City, CA).

In vitro *activity and stability assays.* In order to determine *in vitro* activity of siRNAs, Hepa 1-6 cells were transfected with 10 nmol/l of siRNAs using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), or transfected with 2 μmol/l of Toc-siRNAs without any transfection reagents. The cells were harvested 24 hours after transfection. Total RNA was extracted and the amount of endogenous *apoB* mRNA was measured using qRT-PCR.

In order to study the stability of the siRNAs in serum, (i) siRNA with both strands naked, (ii) siRNA with both strands modified, (iii) siRNA with only the sense strand modified, (iv) Toc-siRNA with both strands naked, and (v) Toc-siRNA with both strands modified (100 pmol each) were incubated at 37 °C in mouse serum for 4, 8, 16, and 24 hours. Aliquots taken at different time points were treated with Proteinase K (Wako Pure Chemical Industries, Osaka, Japan) and frozen in urea Tris-buffered electrophoresis-loading buffer. All samples were subjected to electrophoresis on 2% agarose gels.

Northern blotting. Total RNA was extracted from mice liver using MirVana (Ambion, Austin, TX). Total RNA was condensed with Ethachinmate (Nippon gene) and 2 μg of RNA was separated by electrophoresis on a 14% polyacrylamide-urea gel and transferred to a Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ). The blot was hybridized with a probe of the siRNA antisense sequence which was labeled with fluorescein using Gene Images 3′-Oligolabelling kit (Amersham Biosciences, Piscataway, NJ). The signals were visualized by Gene Images CDP-star detection Kit (Amersham Biosciences, Piscataway, NJ).

Pathological analysis. For pathological analysis of side effects by Toc-siRNA, the liver sample was postfixed in 4% paraformaldehyde/phosphate-buffered saline solution for 6 hour and embedded in paraffin, sectioned at 4-μm thick using a Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany), and then stained with hematoxylin/eosin.

To analyze of liver lipid accumulation, liver samples from apoB-1 and control Toc-siRNA-treated mice were sectioned (4 μm) and fixed in 4% paraformaldehyde/phosphate-buffered saline for 5 minutes, and then stained with filtrated Sudan III (Muto Pure Chemicals, Tokyo, Japan) 37 °C for 30 minutes. Counterstaining of nuclei was performed with Mayer hematoxylin solution (Muto Pure Chemicals, Tokyo, Japan) for 3 minutes.

For pathological analysis of delivery of siRNA to liver, 8 mg/kg Cy3-labeled siRNA with or without α-tocopherol within 0.25 ml of 10% maltose was injected from the tail vein of ICR mouse. One hour after intravenous injection, mouse was killed and liver samples were harvested. Liver samples were fixed in 4% paraformaldehyde/phosphate-buffered saline for 6 hour. Fixed tissue samples were snap-frozen in liquid nitrogen. Frozen tissue sections were prepared and stained with 13 nmol/l Alexa-488 phalloidin (Invitrogen, Carlsbad, CA). The slides were analyzed using LSM 510 confocal microscope (Carl Zeiss MicroImaging, Oberkochen, Germany). Each image comprised a flattened projection of 11 optical images (0.4 μm each) to represent combined fluorescence signals from a 4-μm thick section.

Statistical analysis. Student's *t*-test was used to evaluate differences between siRNA-transfected groups and cells alone *in vitro*, and between Toc-siRNA-injected groups and maltose only injected group *in vivo*.

ACKNOWLEDGMENTS

We thank Tadaaki Ohgi, Nippon Shinyaku, for his technical support. This work was supported by grants from the Ministry of Education, Science and Culture, Japan (#18650103) and the Ministry of Health Labor and Welfare, Japan (#2212065), and a grant from the 21st Century Center of Excellence Program on Brain Integration and its Disorders given to Tokyo Medical and Dental University.

REFERENCES

1. Davidson, BL and Harper, SQ (2005). Viral delivery of recombinant short hairpin RNAs. *Methods Enzymol* **392**: 145–173.
2. McCaffrey, AP, Meuse, L, Pham, TT, Conklin, DS, Hannon, GJ and Kay, MA (2002). RNA interference in adult mice. *Nature* **418**: 38–39.
3. Hino, T, Yokota, T, Ito, S, Nishina, K, Kang, YS, Mori, S *et al.* (2006). *In vivo* delivery of small interfering RNA targeting brain capillary endothelial cells. *Biochem Biophys Res Commun* **340**: 263–267.
4. Zimmermann, TS, Lee, AC, Akinc, A, Bramlage, B, Bumcrot, D, Fedoruk, MN *et al.* (2006). RNAi-mediated gene silencing in non-human primates. *Nature* **441**: 111–114.
5. Yokota, T, Iijima, S, Kubodera, T, Ishii, K, Katakai, Y, Ageyama, N *et al.* (2007). Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C. *Biochem Biophys Res Commun* **361**: 294–300.
6. Spagnou, S, Miller, AD and Keller, M (2004). Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. *Biochemistry* **43**: 13348–13356.
7. Baigude, H, McCarroll, J, Yang, CS, Swain, PM and Rana, TM (2007). Design and creation of new nanomaterials for therapeutic RNAi. *ACS Chem Biol* **2**: 237–241.
8. Rozema, DB, Lewis, DL, Wakefield, DH, Wong, SC, Klein, JJ, Roesch, PL *et al.* (2007). Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes. *Proc Natl Acad Sci USA* **104**: 12982–12987.
9. Sato, A, Takagi, M, Shimamoto, A, Kawakami, S and Hashida, M (2007). Small interfering RNA delivery to the liver by intravenous administration of galactosylated cationic liposomes in mice. *Biomaterials* **28**: 1434–1442.
10. Kim, SI, Shin, D, Choi, TH, Lee, JC, Cheon, GJ, Kim, KY *et al.* (2007). Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol Ther* **15**: 1145–1152.
11. Kumar, P, Wu, H, McBride, JL, Jung, KE, Kim, MH, Davidson, BL *et al.* (2007). Transvascular delivery of small interfering RNA to the central nervous system. *Nature* **448**: 39–43.
12. Kappus, H and Diplock, AT (1992). Tolerance and safety of vitamin E: a toxicological position report. *Free Radic Biol Med* **13**: 55–74.
13. Rigotti, A (2007). Absorption, transport, and tissue delivery of vitamin E. *Mol Aspects Med* **28**: 423–436.
14. Zingg, JM (2007). Vitamin E: an overwiew of major research directions. *Mol Aspects Med* **28**: 400–422.
15. Rose, SD, Kim, DH, Amarzguioui, M, Heidel, JD, Collingwood, MA, Davis, ME *et al.* (2005). Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res* **33**: 4140–4156.
16. Soutschek, J, Akinc, A, Bramlage, B, Charisse, K, Constien, R, Donoghue, M *et al.* (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**: 173–178.
17. Chiu, YL and Rana, TM (2003). siRNA function in RNAi: a chemical modification analysis. *RNA* **9**: 1034–1048.
18. Czauderna, F, Fechtner, M, Dames, S, Aygün, H, Klippel, A, Pronk, GJ *et al.* (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res* **31**: 2705–2716.
19. Miyoshi, K, Tsukumo, H, Nagami, T, Siomi, H and Siomi, MC (2005). Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. *Genes Dev* **19**: 2837–2848.
20. Judge, AD, Bola, G, Lee, AC and MacLachlan, I (2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing *in vivo*. *Mol Ther* **13**: 494–505.
21. Sioud, M, Furset, G and Cekaite, L (2007). Suppression of immunostimulatory siRNA-driven innate immune activation by 2′-modified RNAs. *Biochem Biophys Res Commun* **361**: 122–126.

22. Aftergood, L and Alfin-Slater, RB (1978). Effect of administration of α- and γ-tocopherol on tissue distribution and red cell hemolysis in rats. *Int J Vitam Nutr Res* **48**: 32–37.
23. Wolfrum, C, Shi, S, Jayaprakash, KN, Jayaraman, M, Wang, G, Pandey, RK *et al.* (2007). Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. *Nat Biotechnol* **25**: 1149–1157.
24. Chen, Z, Fitzgerald, RL, Averna, MR and Schonfeld, G (2000). A targeted apolipoprotein B-38.9-producing mutation causes fatty livers in mice due to the reduced ability of apolipoprotein B-38.9 to transport triglycerides. *J Biol Chem* **275**: 32807–32815.
25. Raabe, M, Véniant, MM, Sullivan, MA, Zlot, CH, Björkegren, J, Nielsen, LB *et al.* (1999). Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J Clin Invest* **103**: 1287–1298.
26. Minehira-Castelli, K, Leonard, SW, Walker, QM, Traber, MG and Young, SG (2006). Absence of VLDL secretion does not affect α-tocopherol content in peripheral tissues. *J Lipid Res* **47**: 1733–1738.
27. Food and Nutrition Board, National Academy of Sciences, National Research Council (1989). *Recommended Dietary Allowances* 10th edn. National Academy Press: Washington, DC, pp 99–107.
28. Robbins, M, Judge, A, Liang, L, McClintock, K, Yaworski, E and MacLachlan, I (2007). 2′-O-methyl-modified RNAs act as TLR7 antagonists. *Mol Ther* **15**: 1663–1669.
29. Morrissey, DV, Lockridge, JA, Shaw, L, Blanchard, K, Jensen, K, Breen, W *et al.* (2005). Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* **23**: 1002–1007.